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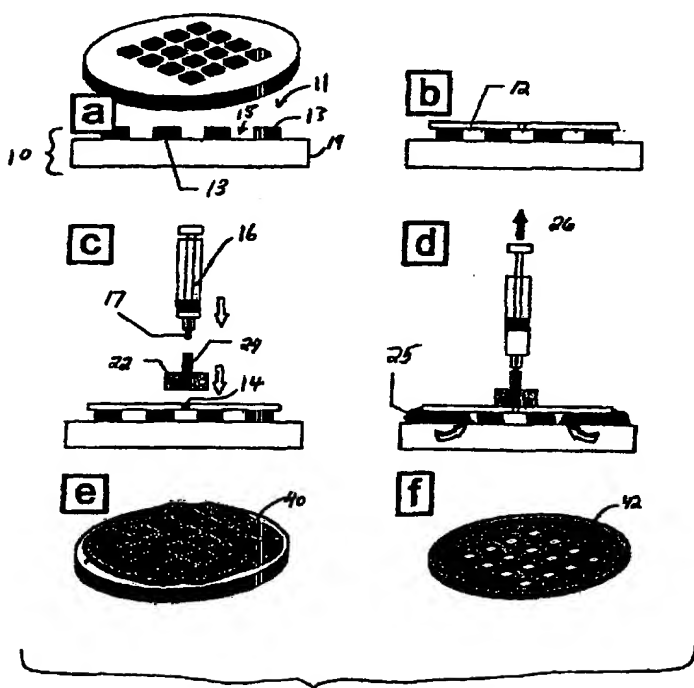
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[Continued on next page]

(54) Title: ELASTOMERIC STENCILS FOR MICROPATTERNING CELLS



(57) Abstract: The present invention provides new fabrication methods and techniques for making and using stencils to micropattern cells. These techniques may allow the fabrication of thinner stencils. The invention provides a system for selectively applying cells to predetermined regions on a substrate. A stencil is positioned adjacent to a substrate to cover some portions of the substrate, while allowing other portions of the substrate to remain uncovered. The stencil may have one or more channels passing through the stencil, and in certain cases, the channels may have a cross-sectional dimension of less than 1 mm. The stencil may be applied to a cell culture substrate in such a way as to form a microscopic cellular pattern on the substrate, and the stencil may form a seal or other watertight contact with the substrate in some cases. In one embodiment, the stencil may be applied to a substrate before seeding. Cells are applied to the uncovered portions of the surface, then the stencil may be removed if desired. The stencil may be fabricated, for example, by injecting an elastomeric precursor into a mold, or by curing an elastomeric precursor while applying pressure. In some embodiments, the surface of the stencil may also be oxidized, or it may be treated in such a way as to render the surface of the stencil more hydrophilic, for

example, by treating the surface using oxygen-plasma treatment.



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**ELASTOMERIC STENCILS FOR MICROPATTERNING CELLS****BACKGROUND****5    Field of the Invention**

This invention generally relates to the micropatterning of surfaces and, in particular, to the micropatterning of cells on surfaces.

**Description of the Related Art**

10        Cell adherence on substrate surfaces, particularly surfaces used for cell culture such as glass or plastic, is necessary in many instances for the study of cells in furthering applications such as tissue engineering, biosensors, etc. Cell patterning, i.e. placing cells in discrete portions of a surface, has been provided by photolithography. Although the technology of photolithography is very highly developed, it presents  
15        several disadvantages. Photolithography presents harsh conditions which can destroy the cells themselves. Clean-room facilities and other complex equipment are also required and such facilities and equipment are not readily accessible to most biologists. Photolithography is not amenable to controlling the molecular properties of a surface required for many sophisticated cell-biological experiments. In addition,  
20        photolithography modifies a surface only at the beginning of an experiment. Once cells are deposited, photolithography cannot be used to make further surface modifications.

International Patent Application WO 99/54786, published October 28, 1999, entitled "Elastomeric Mask and Use in Fabrication of Devices, Including Pixelated Electroluminescent Displays," describes an elastomeric mask that can be used to  
25        fabricate certain device components. International Patent Application WO 01/70389, published September 27, 2001, entitled "Cell Patterning Technique," describes an elastomeric mask for cell patterning.

**SUMMARY OF THE INVENTION**

30        This invention relates to the micropatterning of cells on surfaces. The subject matter of this application involves, in some cases, interrelated products, alternative

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solutions to a particular problem, and/or a plurality of different uses of a single system or article.

In one aspect, the invention provides a method. One method includes the steps of providing a substantially enclosed mold constructed and arranged to produce an article having a plurality of channels passing through the article, where at least one of the plurality of channels having a cross-sectional dimension of less than 1 mm. The method also includes the steps of injecting a precursor of a flexible polymer into the mold, and curing the precursor to produce a flexible polymeric article.

A method is provided in another embodiment of the invention, which includes the steps of providing a mold able to produce an article having a plurality of channels passing through the article, where at least one of the plurality of channels having a cross-sectional dimension of less than 1 mm. The method also includes the steps of adding a precursor of a flexible polymer to the mold, and applying pressure to the precursor while curing the precursor to produce a flexible polymeric article.

The method, in yet another embodiment, includes the step of treating at least a portion of a surface of a flexible polymeric article using oxygen-plasma treatment. The article has a plurality of channels passing through the article, where at least one of the plurality of channels having a cross-sectional dimension of less than 1 mm.

In another aspect, the invention provides a flexible polymeric article. One article comprises a first surface, an opposing second surface, and a plurality of channels connecting the first surface with the second surface. At least one of the plurality of channels has a cross-sectional dimension of less than 1 mm and a length of less than about 25  $\mu\text{m}$ .

In another aspect, the invention is directed to a system for molding an article. The system includes components, which, when assembled, define a molding space, which can be enclosed. The molding space can include openings from which excess mold and material can escape, or through which precursor material can be introduced. The enclosed molding space can include interior surfaces that are patterned, for example, with protrusions that extend partially or completely across the molding space, allowing molding of an article containing channels therethrough. The molding space can include at least one very narrow dimension and in a set of preferred embodiments, coincident with dimensions of molded articles described herein.

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In another aspect, the invention is directed to a method of making any of the embodiments described herein. In yet another aspect, the invention is directed to a method of using any of the embodiments described herein.

Other advantages, novel features, and objects of the invention will become  
5 apparent from the following detailed description of non-limiting embodiments of the invention when considered in conjunction with the accompanying drawings, which are schematic and which are not intended to be drawn to scale. In the figures, each identical or nearly identical component that is illustrated in various figures typically is represented by a single numeral. For purposes of clarity, not every component is  
10 labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. In cases where the present specification and a document incorporated by reference include conflicting disclosure, the present specification shall control.

15

### BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying drawings in which:

Fig. 1(A-F) schematically illustrate a stencil fabrication process using an  
20 injection technique;

Fig. 2 (A-F) schematically illustrate a stencil fabrication process using an applied pressure;

Fig. 3 (A-D) illustrate photomicrographs of various cellular micropatterns; and

Fig. 4 (A-D) illustrates photomicrographs of stenciled cell cultures on various  
25 substrates.

### DETAILED DESCRIPTION

The present invention provides new fabrication methods and techniques for making and using articles such as stencils to micropattern cells. The fabrication  
30 techniques of the invention can be used to make stencils and other articles. Stencils or articles of the invention can be made according to these or other techniques. Some techniques may allow the fabrication of thinner stencils or other articles containing

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channels. The invention provides a system for selectively applying cells to predetermined regions on a substrate. A stencil is positioned adjacent to a substrate to cover some portions of the substrate, while allowing other portions of the substrate to remain uncovered. The stencil may have one or more channels passing through the  
5 stencil, and in certain cases, the channels may have a cross-sectional dimension of less than 1 mm. The stencil may be applied to a cell culture substrate in such a way as to form a microscopic cellular pattern on the substrate, and the stencil may form a seal or other watertight contact with the substrate in some cases. In one embodiment, the stencil may be applied to a substrate before seeding. Cells (for example, hepatocytes,  
10 fibroblasts, or keratinocytes, or any other adherent type of cell) are applied to the uncovered portions of the surface, then the stencil may be removed if desired. The stencil may be fabricated, for example, by injecting an elastomeric precursor into a mold, or by curing an elastomeric precursor while applying pressure. In some embodiments, the surface of the stencil may also be oxidized, or it may be treated in  
15 such a way as to render the surface of the stencil more hydrophilic, for example, by treating the surface using oxygen-plasma treatment.

The stencil (or similar articles) may have any shape or structure able to produce a micropattern of cells on a substrate. The stencil may include, for example, a membrane containing holes, channels, or other similar structures, formed in or through  
20 the membrane. In one embodiment, there is a channel within the stencil, and preferably a plurality of channels within the stencil. The stencil can comprise first and second opposing surfaces where the channel passes through the stencil, connecting the first surface with the second surface. The channel may function to expose certain portions (a second portion) of the surface of the article, whereas a first portion of the article is  
25 shielded due to conformal contact of the article with the masking system. By "conform" it is meant to define essentially continuous contact between the stencil and the portions of the substrate to be patterned. This is to be distinguished from, for example, a metal screen or a rigid polymer, each of which can contact a surface to be masked, but which may not be flexible enough to conformally contact the surface. The  
30 flexibility of the stencil may be provided by the use of, for example, an elastomeric stencil. In one embodiment, the stencil can shield selected portions of the substrate by being brought into contact with those portions.

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The stencil (or similar articles) may be made of a polymeric material, and flexible polymeric materials are preferred in some embodiments of the invention. In one set of embodiments, the stencil (or other articles made in accordance with the invention), comprises an elastomeric material. A variety of elastomeric materials may be suitable, especially polymers of the general classes of silicone polymers, epoxy polymers, and acrylate polymers. Epoxy polymers are characterized by the presence of a three-member cyclic ether group commonly referred to as an epoxy group, 1,2-epoxide, or oxirane. For example, diglycidyl ethers of bisphenol A may be used, in addition to compounds based on aromatic amine, triazine, and cycloaliphatic backbones. Another example includes the well-known Novolac polymers. Examples of silicone elastomers suitable for use as component 12 include those formed from precursors including the chlorosilanes such as methylchlorosilanes, ethylchlorosilanes, phenylchlorosilanes, and the like. One useful silicone elastomer is polydimethylsiloxane. The stencil may be reusable in some cases.

In some cases, the stencil or other articles of the invention may contain additional features, such as structures that provide for mechanical stability. For example, any structure able to prevent the stencils from holding or sticking to themselves or to other surfaces may be used, such as ribs or braces. As examples, an annulus of material may be bonded to the stencil as shown in Fig. 1F, or ribs or other materials may be used to, for example, stiffen the stencil or increase structural integrity.

The stencil may be of a variety of shapes or dimensions. The stencil may be, for example, circular, rectangular, square, triangular, oval, or irregularly shaped. In some embodiments, the stencil has a thickness of no more than about 1 mm; in other embodiments, no more than about 500  $\mu\text{m}$ ; in still other embodiments, no more than about 200  $\mu\text{m}$ ; in still other embodiments, no more than about 100  $\mu\text{m}$ ; and in other embodiments, no more than about 50  $\mu\text{m}$  or 25  $\mu\text{m}$ . In other embodiments, thinner stencils may be created. For example, the thickness of the stencil may be less than 20  $\mu\text{m}$ , less than 15  $\mu\text{m}$ , less than 10  $\mu\text{m}$ , or less than 5  $\mu\text{m}$  in some cases.

Depending on the number of cells to be deposited, the channels or holes of the stencil or other article can have a diameter of less than about 3 mm, less than about 1 mm, less than about 500  $\mu\text{m}$ , less than about 250  $\mu\text{m}$ , less than about 100  $\mu\text{m}$ , less than about 50  $\mu\text{m}$ , less than about 25  $\mu\text{m}$ , less than about 10  $\mu\text{m}$ , less than about 5  $\mu\text{m}$ , less



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than about 2 mm, down to less than about 1.5  $\mu\text{m}$  or 1  $\mu\text{m}$ . The channels may connect one side or surface of the stencil with an opposed side or surface. The channels do not necessarily connect one side or surface of the stencil with the opposed side or surface; in some embodiments, at least a portion of the channels may not fully extend across the stencil. The channels may have any shape (e.g., having a rectangular, circular, or square cross-section), and any orientation through the stencil. In some cases, the channels may be formed directly or linearly through the article. The new molding techniques of the present invention may allow for thinner stencils to be created, where the minimum thickness of the stencil is limited only by its structural integrity of the stencil or other such article. The minimal structural integrity may be determined by those of ordinary skill in the art. It will be appreciated that the minimum thickness is, in part, controlled by the formulation of the prepolymer. For example, polymers having better cohesion, higher molecular weight, stronger interchain binding, higher degrees of crosslinking, or those polymers that can be made as thin films may result in polymeric stencils having greater structural integrity, such that the stencil may be thinner.

The stencil (or other article of the invention) may have any number of channels or holes. For example, the stencil may have 1 channel, 9 channels, 25 channels, 100 channels, 300 channels, 500 channels, or 750 channels, depending on the application. In some applications, large numbers of channels are possible, for example, thousands, tens of thousands, hundreds of thousands, or millions of channels. Of course, the number of channels and the shape of channels can be varied by any method known to one of ordinary skill in the art.

In some embodiments, the size or shape of the channel or hole may be selected to allow only the entry of one or a small number of cells within the channel. For example, if the diameter of the channel is roughly the diameter of a cell in suspension, or even 2-3 times as big as the diameter of the cell in suspension, it may fit only one or a few cells. Thus, the resolution of cells may depend on factors such as the height-to-width ratio of the holes or channels, or the thickness of the stencil. For example, in a thinner stencil or article, the walls may be easier to wet or to fill with cells, the channel-to-channel spacing may be smaller, or the creation of the master may be easier. Thus, for example, an approximately 100-micron thick stencil, containing roughly 40-micron

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diameter channels, each separated on the average by about 250 microns, may allow for the creation of single-cell micropatterns of certain cells such as keratinocytes (Fig. 4C).

Although the above description of sizes and numbers of channels or holes, and other features are described in connection with a stencil, the invention involves articles, production of articles, and use of articles that are not limited to stencils. All of the various embodiments described with respect to stencils can apply to other articles and vice versa as well, in accordance with the invention.

In one embodiment, the stencil comprises a polymer, preferably a flexible polymer. A preferred polymer is a polymeric elastomer that can form a seal against the surface of the article, preferably a conformal seal. "Seal" in this context means that when the stencil is sealingly engaged with a surface or substrate and a fluid is applied to the masked surface, the fluid is allowed to contact only those portions of the masked surface in register with channels of the stencil and the fluid does not pass under the stencil and contact shielded portions of the surface covered by solid portions of the stencil, so long as the fluid does not degrade the stencil or the surface to be patterned (in which case fluid could pass under the stencil due to degradation of the stencil and/or surface). For example, the seal can prevent a protein solution from seeping under the stencil. "Sealing" in this context is to be distinguished from the operation of other rigid or flexible stencils that may be brought into conformal contact with a surface or other substrate, but that can not seal against the surface. It is a feature of the invention that stencils of the invention can form a seal against a substrate surface in the absence of any clamping apparatus or other apparatus used to apply a force against the stencil in a direction of the substrate surface. Where elastomeric surfaces are used, and the elastomeric surface and substrate surface to be masked are clean, sealing can occur essentially instantaneously upon contact without application of significant pressure, and sealing can be maintained without maintenance of any pressure. This sealing is reversible, that is, the stencil can be removed from the substrate surface by being peeled off, and can be reused on the same or a different substrate surface.

In some embodiments, the surface of the stencil (or other article) may be oxidized, for example, using chemical oxidation or exposure of the surface to a plasma. For example, exposure of the stencil to the plasma may result in exposed oxygen atoms that become hydroxyl groups able to carry a negative charge. Pre-oxidizing one or

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more surfaces of the stencil can render the stencil more hydrophilic, for example, allowing easier wetting of an aqueous solution through channels in the stencil, or allowing easier deposition of cells within the stencil.

In one embodiment, a stencil or other article of the invention may be treated in  
5 such a way as to make the surface more hydrophilic or hydrophobic. As examples of this, the stencil may be treated in such a way as to prevent the binding of cells to the surface, or to prevent gas bubbles from attaching to the surface of the stencil, for example, when the stencil is covered with water or media. Any suitable technique may be used for treating the surface. For example, one suitable treatment to render the  
10 surface more hydrophilic is to treat the stencil with a plasma such as an oxygen plasma.

The stencil or other article may be used with a wide range of substrates or surfaces, for example, biocompatible polymers, microelectronic or semiconductor chips, gels, tissue culture dishes, biological tissues, polymers such as polystyrene, a humid surface such as a collagen gel, or the like. The substrate may include metal  
15 oxides, such as silica, alumina, quartz, glass, and the like or derivatives thereof, or the substrate may include metals such as gold, silver and copper. In one set of embodiments, the substrate may include a semiconductor material, such as silicon, GaAs, or  $\text{Si}_3\text{N}_4$ . In some embodiments, the substrate or surface may be coated, for example, with a hydrophilic material. In one example, illustrated in Fig. 4D, a  
20 micropattern of hepatocytes is created on a collagen gel. In this embodiment of the invention, contact between the stencil and the collagen gel may be tight enough to prevent cell suspension from falling into it, but loose enough for attached cells to send filopodia into it. In this example, the stencil was removed 24 hours after seating, and a second layer of collagen gel was added after the patterning step to produce a "sandwich  
25 configuration" of cells between two layers of collagen gels. Heterogeneous surfaces can also be patterned using the present invention, such as a microelectrode. As one example, Fig. 4A illustrates micropatterned cells a gold/chromium microelectrode pattern on glass.

Due to the flexibility of the stencil, the substrate may be either a planar or non-  
30 planar surface, such as a glass cylinder, a Pasteur pipette. In one embodiment, the surface is an irregular surface, for example, in an implantable structure. For example, the micropattern of fibroblasts shown in Fig. 4B was created on an approximately 6

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mm diameter glass cylinder (note that only few rows of the micropattern in Fig. 4B are in focus, due to the curvature of the surface). Additionally, the flexibility of the stencil may facilitate release of the stencil from the substrate or surface.

Any cell may be micropatterned with the present invention, for example  
5 hepatocytes, fibroblasts, or keratinocytes. Virtually any adherent cell type able to adhere onto homogeneous or heterogeneous surfaces or substrates may be used. In some cases, micropatterns of cells may be created to simulate physiological conditions, or to enhance cell function. As one example, a micropattern of hepatocytes and fibroblasts may be created using the methods of the invention to enhance hepatocyte  
10 function. Cells may also be micropatterned to study various phenomena, for example, cell growth, migration, differentiation, or wound healing.

To achieve a desired micropattern of cells, the methods of the present invention do not necessarily depend on such factors as cell type or medium formulation; thus, these variables may be changed between applications. For example, non-confluent  
15 islands or serum-free media may be desired for certain experiments, whereas multilayers of cells or serum-containing media may be required for others.

In some embodiments of the invention, the stencil is created in a "mold." Articles other than stencils can be created in molds in accordance with the invention and it should be understood that where description of creation of a stencil in a mold as  
20 described herein, the invention encompasses formation of essentially any article in a mold. A prepolymeric substance is placed in the mold, then the substance is treated such that the prepolymeric substance forms an article of the invention. Any technique may be used for creating the mold. For example, the mold, or a pattern or micropattern in the mold, may be microfabricated or machined, for example, using various  
25 photolithographic or etching techniques. In some embodiments, the mold is open to the atmosphere; however, in other embodiments, the mold may be enclosed or substantially enclosed, for example, to control environmental conditions within the mold. Of course, many stencils may be replicated many times from the same mold using techniques of the invention.

30 As one example, a master wafer for use in the mold may be microfabricated containing photoresist posts having predetermined shapes or heights. The posts may become the "supporting columns" of a chamber, if an upper surface or a "roof" is added

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to the master. If the posts do not extend to reach the upper surface, then a channel that does not cross to an opposed surface may result. The posts may be photolithographically defined on any suitable substrate, for example, a semiconductor wafer such as a  $\text{Si}_3\text{N}_4$ -coated silicon wafer.

5 Any method may be used to create a stencil within the mold. For example, a prepolymer may be added to the mold, then the mold cured. The mold may be removed before or after curing, depending on the strength of the prepolymer and the nature of the process. One example method of creating a stencil in the mold is as follows, and is described with reference to Fig. 1. A mold 10 may be prepared including, for example,  
10 a patterned surface 11 (which, as illustrated, includes a plurality of regularly-spaced protrusions 13 defining intervening indentations 15) created using, for example, photolithographic techniques. The mold can be provided with an upper wall or surface 12, defining an interior upper surface which can rest on upper surfaces of protrusions 13, as shown. The upper surface 12 of the mold 10 may be formed of any material, for  
15 example, glass, plastic, rubber a translucent or transparent adhesive film. Upper surface 12 may be rigid or semi-rigid, depending on the application. In some embodiments, upper surface 12 may be a stack or include several layers of material. Suitable materials for the upper surface include, for example, plastic, glass, or rubber.

In some embodiments, one or more openings 14 may be formed in upper  
20 surface 12, for example, to allow for a fluidic connection to a syringe 16 (Fig. 1C) or another fluid transport mechanism, such as a valve, a pump, a tube, or a dispensing unit. Prepolymer 25 may then be injected into the mold. In one embodiment, prepolymer 25 is added through one or more openings, for example, using a suitable pressure. In another embodiment, prepolymer may be dispersed at the edges of the  
25 molding chamber, or at predetermined loading sites around the molding chamber, and suction applied to the opening to generate flow of polymer into or through the mold, directed towards the hole or holes (Fig. 1D). The upper surface 12 may then be peeled off or otherwise removed, for example, before or after curing of the prepolymer (Fig. 1E). As used herein, the term "inject," when used in conjunction with the mold, refers  
30 to the introduction of a substance into the mold under pressure (i.e., under positive or negative pressure).

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In another set of embodiments, the prepolymer may be dispensed within the mold before adding an upper surface, in embodiments including an enclosed mold. With reference to the example illustrated in Fig. 2, after the prepolymer has been added to the mold, upper surface 12 may be added and pressed tightly against the bottom surface 19, which may squeeze out excess prepolymer 25 from the mold, for example, through predefined openings in the mold, or between the upper and lower surfaces. For example, pressure may be applied until the master and the transparency come into intimate contact (Fig. 2D). The pressure may be applied from the top surface of the mold, the bottom surface of the mold, or through a combination of pressures applied to both surfaces. After curing, upper surface 12 may be separated from bottom surface 19 and the fabricated article 40 released (alternatively, the article may be removed from the mold before or during the curing process).

Additional structures may be added to the stencil or article at any point, for example, before, during, or after curing. For example, structures that prevent bending or distortion of the stencil may be added, as previously described. As one example, ribs, braces, or an annulus of material 42, as shown in Fig. 1F, may be added to the stencil.

The prepolymer may be cured or hardened by any suitable technique, depending on the nature of the prepolymer. For example, if the prepolymer comprises PDMS, the prepolymer may be cured or hardened within the mold by heating the prepolymer to the curing temperature, for example, to a temperature of at least about 65 or 70 °C. With other materials, other curing techniques may be required, for example, exposure of the article to a gas or a curing solution. The time chosen may be any length of time sufficient to allow the prepolymer to cure, for example, for 2 hours or 4 hours. In addition, to minimize bleaching of monomers, which could affect cell function, the prepolymer may be overcured, for example, for 8 hours, 10 hours, 12 hours, or even longer in some cases.

After curing, the stencil or article may be cleaned by any suitable solvent, for example, to remove unreacted prepolymer. In one embodiment, the article may be cleaned using an organic solvent such as acetone, ethanol or isopropanol. In some embodiments, the prepolymer may be cleaned using a plasma, for example, oxygen plasma treatment (which may also affect hydrophilicity of the stencil, as further

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described below), or a combination of techniques may be used. The various plasma treatments may also remove undesired contaminants from the stencil, in some cases.

Certain problems may occur if bubbles of gas become trapped within the mold, especially if the prepolymer contains hydrophobic surfaces. For example, when the stencil or article is applied onto a substrate and covered with water or media, a bubble of gas may become trapped within a stencil channel, for example, in a channel with a lateral dimension of less than 100 microns. One method of facilitating the release of gas bubbles from the stencil is to treat the surface of the stencil using oxygen plasma so as to render it hydrophilic. Other methods include placing the stencil in a low vacuum (e.g., at a pressure of approximately 15 mTorr or 20 mTorr), adding ethanol or another solvent to the solution that is able to lower surface tension, allowing the bubble sufficient time to dissolve in the liquid (e.g., about 24 hours, about 36 hours, or about 48 hours), or adding a surfactant to facilitate wetting (e.g., Tween, or a protein solution, such as a bovine serum albumin solution). In some cases, a combination of these methods may be used. For example, the surface may be treated by ethanol or isopropanol, followed by vacuum degassing.

The stencil may also be sterilized using any suitable technique known to those of ordinary skill in the art, for example, under ultraviolet light, or by immersion in a sterilizing solution such as ethanol in water.

The stencil or other article of the invention may then be used to micropattern cells on a surface, as previously described. Various techniques may be used to create the micropattern. For example, a stencil may be sealingly bonded onto a substrate and manually pulled off after seeding of cells in some embodiments, or the stencil may left in place in other embodiments (for example, if the stencil comprises a nontoxic or noncytotoxic material). It is to be understood that the order of steps for shielding using the stencil, application of external agents and media, application of cells, and removal of the stencil (if desired) can be varied to obtain a desired result.

In one embodiment, the cells may be seeded at 25,000-50,000 cells/cm<sup>2</sup> to obtain a confluent monolayer of cells within each "island" or other pattern. Depending on the cell type, higher or lower cell densities may also be used, for example, a density of 100 cells/cm<sup>2</sup>, 10,000 cells/cm<sup>2</sup>, 100,000 cells/cm<sup>2</sup>, or even 500,000 cells/cm<sup>2</sup> or 1,000,000 cells/cm<sup>2</sup>. The cells may be seeded for any suitable length of time, for

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example, a fixed length of time (e.g., 1 hour, 2 hours, 3 hours, or 4 hours), or for a time that allows a certain percentage of cells to attach to the substrate (e.g., when 50% or 70% of the cells have become attached to the surface). During sedimentation of the cell suspension onto the stencil, vibrating or shaking the dish may displace the cells at the top of the stencil thus resulting in an effective higher cell density. In some embodiments, cells may be seeded at low densities and be allowed to grow, for example, until confluent or a certain percentage confluence is reached (e.g., about 70% confluent), or until a predetermined monolayer micropattern is achieved. In other embodiments, for example, where a monolayer of patterned cells is desired, high seeding densities may be used.

In embodiments where the cells may make strong contacts with the stencil or the substrate within short periods of time, the removal of the stencil may not result in damage to the cells immediately adjacent to the cell walls if the stencil is removed before attachment occurs, or, for "contact guidance" cells, the cells attached to the stencil may refuse to make contact with the cells attached to the surface and vice versa. "Contact guidance" cells are cells that generally are able to respond to the topographic or local features of their environment.

In some embodiments, areas that remain free of cells after removing the stencil or other article may then be seeded with a second type of cell. For example, a first area may be seeded with hepatocytes and a second area contiguous with the first area may be seeded with fibroblasts. In certain embodiments, this process may be repeated several times, thus allowing the micropatterning of third, fourth, fifth, sixth, or even more areas or types of cells.

The resulting pattern of cells can be used for a variety of applications, including observing cell growth and spreading, cell migration, wound healing, chemotaxis, haptotaxis, morphogenesis, developing biology artificial organ studies and the patterning of multiple cell types. In addition, cell patterning can have long range applications in the study of regeneration, partial regeneration or healing of human organs and wounds, i.e. tissue engineering. Other applications involve biosensors.

The function and advantage of these and other embodiments of the present invention will be more fully understood from the examples below. The following



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examples are intended to illustrate the benefits of the present invention, but do not exemplify the full scope of the invention.

### Example 1

5           This example details the microfabrication of a master used to produce a mold in one embodiment of the invention.

          A 50-300 micron layer of suitable photoresist was spun on a TET-grade silicon wafer coated with 150 nm of  $\text{Si}_3\text{N}_4$ , dried at 95 °C on a hot plate, and exposed to collimated UV light through either a chrome mask or a high-resolution transparency  
10   mask pressed against the photoresist layer. The unexposed areas were dissolved in propylene glycol methyl ether acetate. The transparency masks were printed either at 3386 or 5000 dots per inch (about 7.5 micron or 5 micron-diameter dots, respectively). The features on the masks were designed to produce posts of various shapes and sizes on the master.

15           The master was then used to produce elastomeric stencils, as discussed in other examples.

### Example 2

          In this example method, illustrated in Fig. 1, a stencil fabricated using an  
20   injection molding technique is described.

          A molding chamber fabricated using techniques similar to those described in Example 1 was filled with PDMS prepolymer. The molding chamber includes a bottom surface 10 having protrusions 13, and an upper surface 12. (also in Ex. 3) In this example, the tissue culture substrates were fabricated to be circularly symmetric,  
25   but other shapes, of course, are possible.

          PDMS prepolymer was prepared as a mixture of two components, mixed at 10:1 by weight. The mixture was degassed to eliminate bubbles created during mixing by placing the PDMS prepolymer in a dedicated desiccator at low vacuum (e.g., about 30 Torr). Breaking the vacuum periodically was used to facilitate removal of bubbles  
30   from the surface of the mixture.

          The roof or upper surface of the mold 12 was made with a thin adhesive film applied to the master (Figs. 1A and 1B). For spaced columns, where sagging of the

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film onto the wafer could occur, pressure was applied to the upper surface, for example, using a 1-mm thick glass plate. The adhesive upper surface 12 was perforated with a small hole 14. A connector between the hole 14 and a syringe 16 mounted with a fitting 17 was made in this example with a flat piece of PDMS 22 in which a piece of silicon tubing 24 had been embedded. The flat PDMS piece was then positioned onto upper surface 12 so that the center of silicon tubing 24 coincided with hole 14, and the fitting (with the mounted syringe) was fitted into the protruding end of silicon tubing 24 (Fig. 1C).

Next, PDMS prepolymer 25 was poured onto the perimeter of the chamber (for example, a chamber in the shape of a 2-inch diameter disk) and was introduced or drawn into the molding chamber 10 by applying suction pressure 26 from the hole (Fig. 1D). When the chamber was substantially filled with PDMS, it was placed in an oven at 65-70°C for about 12 hours (including 8 hours overcuring to minimize bleaching of monomers, which may affect cell function). After curing was completed, the PDMS connector was removed, and the chamber was opened by removing the upper surface 12 (i.e., the adhesive film) with tweezers (Fig. 1E).

Subsequently, a thick (about 3 mm) PDMS annulus was slightly wetted with PDMS precursor and the bottom was placed on top of the open chamber. The assembly was cured again to cause the PDMS annulus to bond with the stencil. (Alternatively, the PDMS annulus could be bonded after separating the stencil from the master). The stencil was then peeled off with the help of tweezers under a dissection microscope to avoid tearing of small features (Fig. 1F).

Thus, this example illustrates a technique for using an injection process to fabricate an elastomeric stencil.

### Example 3

This example, illustrated in Fig. 2, describes a method of using a compression-molding process to realize a stencil.

In this example, a master (Fig. 2A), fabricated using techniques similar to those described in Example 1, was covered with a prepolymer (Fig. 2B) and then a multilayer stack was used to form the mold chamber. INSERT AA A transparency film 12 was carefully lowered onto the prepolymer, allowing surface tension to pull the

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transparency into intimate and continuous or sealing contact with the prepolymer mixture, for example, to prevent any bubbles from forming at the interface (Fig. 2C). The flexibility of the film allowed for easier removal from PDMS molds after curing. The master/prepolymer/transparency stack was the clamped within a sandwich that  
5 included several layers, including flat aluminum plates (top and bottom, a rigid wafer, and a rubber sheath (top only), as shown in Fig. 2D). The top and bottom aluminum plates provided uniform force 32 onto the stack from both sides. The rubber sheath provided a mechanical buffer layer between the top aluminum plate and rigid wafer, insuring uniform pressure on the wafer and preventing cracking from non-uniformities  
10 in the aluminum plate. The polished rigid wafer produced a flat surface on top of the cured molds.

The clamped PDMS prepolymer sandwich was then cured for about 3 hours at 100°C on a hot plate. After curing, all layers were removed except the transparency layer. The flexible transparency was then removed (Fig. 2E) and the think PDMS  
15 replica was peeled off from the master (Fig. 2F).

This example therefore illustrates a technique to fabricate an elastomeric stencil using pressure.

#### Example 4

20 This example illustrates the use of a stencil of the invention to pattern cells on a substrate.

After separating it from the master, the stencil was washed with acetone and ethanol, dried and gently pressed against the tissue-culture surface of interest. In some cases, the stencil was cleaned and rendered hydrophilic in an oxygen plasma (200 W, 5  
25 min) prior to applying it to the tissue-culture surface. The stencil/substrate assembly was then fully covered with deionized water either in air or in 100% CO<sub>2</sub>. To deliver water under CO<sub>2</sub>, the stencil-covered substrate was placed at the bottom of a beaker (about 25 cm high) that was continuously flushed with CO<sub>2</sub>. Since CO<sub>2</sub> is heavier than air, this method ensured that the gas contacting the stencil was CO<sub>2</sub>. After delivering  
30 the water, the stencil was brought out to air, which resulted in dissolution of the bubbles into the water. In cases where sterility was desired, the whole procedure could be performed inside a tissue-culture hood in sterile conditions.

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To remove bubbles that were trapped on the sides of the chamber, ethanol was squirted directly onto the stencil/substrate under running water carefully avoiding static exposures to ethanol. The stencil/substrate assembly with fully wetted walls was sterilized by overnight UV light exposure in a tissue-culture hood. Prior to seeding, the stencil/substrate assembly was washed twice with medium to fully substitute the water in the wells with medium.

Primary rat hepatocytes were isolated and seeded and cultured in high-glucose Dulbecco-modified Eagle's medium, supplemented with 0.5 Units/L insulin, 14 ng/ml glucagon, 7.5 g/ml hydrocortisone, 20 ng/ml epidermal growth factor, and 10% fetal bovine serum. Mouse 3T3-J2 fibroblast were cultured in high-glucose (DMEM) supplemented with 10% calf serum and 2% penicillin/streptomycin mixture. Serum was heat-inactivated at 56°C for 30 min before being added to DMEM. Before seeding, fibroblasts were detached from the tissue-culture substrate with trypsin-EDTA, centrifuged at 100 rpm for 5 min and resuspended at 1 millionth cells/ml. Keratinocytes were isolated from human neonatal foreskins and cultured on a fibroblast feeder layer in keratinocyte basil medium. All cells were cultured in tissue-culture grade polystyrene petri dishes and incubated at 37°C in a humidified mixture of 9% air and 10% CO<sub>2</sub>. All percentages are by volume.

Collagen type I was isolated from Lewis rat tail tendons and dissolved (about 1.2 g/L) in HCl 0.1 mM (pH = 4) to prevent gel formation. For each petri dish, 2 ml of the gelling solution composed of 9:1 collagen type I and concentrated DMEM was added and incubated for 1 hour at 37 °C to cause gelling. After gelling, and before applying the PDMS stencil, any excess medium was carefully aspirated from the dish. The hepatocyte cultures were covered with a second layer of 2 ml of freshly prepared gelling solution and incubated overnight. Finally, 2 ml over medium were added to each dish.

After the PDMS stencil was applied to the tissue-culture substrate, covered with deionized water, and bubbles were eliminated, water was substituted twice by the seeding medium prior to seeding. After adding the cell suspension, the stencil/substrate assembly was incubated at 37 °C for periods ranging from 2-48 hours to allow for cell attachment and spreading. After peeling off the stencil, the substrate was washed twice

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with seeding medium to remove unattached cells, thus resulting in a substrate with a micropattern of cells.

As a toxicity test, the same experiments were performed, but the stencils were not removed, or were removed after 48 hours. No change in cell function was observed  
5 in either case, thus indicating the nontoxicity of the stencils.

Thus, this example illustrates the use of a stencil to produce a micropattern of cells on a substrate.

#### Example 5

10 In this example, illustrated by the sequence of photomicrographs in Fig. 3, the depiction of a cellular micropattern by means of a 100-micron thick stencil containing 140 micron-side squares separated by 100 microns is described.

The starting substrate was a tissue-culture-grade polystyrene petri dish and the cells were fibroblasts. A 100-micron thick stencil containing 140 micron-side squares  
15 separated by 100 microns was prepared using a technique similar to those described in Examples 1 and 2.

After applying the stencil to the polystyrene petri dish under dry conditions, water was added and the bubbles were removed. Prior to cell seeding, water was substituted by medium (Fig. 3A). Then, the fibroblast suspension was added (Fig. 3B),  
20 and, after allowing about 2 hours for the fibroblasts to attachment and spread (Fig. 3C), the stencil was carefully removed, resulting in a micropattern of fibroblasts on the polystyrene petri dish (Fig. 3D).

While several embodiments of the invention have been described and illustrated  
25 herein, those of ordinary skill in the art will readily envision a variety of other means and structures for performing the functions and/or obtaining the results or advantages described herein, and each of such variations or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art would readily appreciate that all parameters, dimensions, materials, and configurations  
30 described herein are meant to be exemplary and that actual parameters, dimensions, materials, and configurations will depend upon specific applications for which the teachings of the present invention are used. Those skilled in the art will recognize, or

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be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention  
5 may be practiced otherwise than as specifically described. The present invention is directed to each individual feature, system, material and/or method described herein. In addition, any combination of two or more such features, systems, materials and/or methods, if such features, systems, materials and/or methods are not mutually inconsistent, is included within the scope of the present invention.

10 In the claims (as well as in the specification above), all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," and the like are to be understood to be open-ended, i.e. to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States  
15 Patent Office Manual of Patent Examining Procedures, section 2111.03.

What is claimed is:

## CLAIMS

1. A method, comprising:
  - providing a substantially enclosed mold constructed and arranged to  
5 produce an article having a plurality of channels passing through the article, at  
least one of the plurality of channels having a cross-sectional dimension of less  
than 1 mm;  
injecting a precursor of a flexible polymer into the mold; and  
curing the precursor to produce a flexible polymeric article.
- 10 2. The method of claim 1, wherein the channel connects a first surface with an  
opposing second surface of the article.
3. The method of claim 1, wherein the mold comprises a photoresist surface.
- 15 4. The method of claim 1, wherein the mold comprises a semiconductor wafer.
5. The method of claim 1, wherein the mold is produced using photolithography.
- 20 6. The method of claim 1, wherein the injection is performed under negative  
pressure.
7. The method of claim 1, wherein the injection is performed under positive  
pressure.
- 25 8. The method of claim 1, wherein the precursor comprises PDMS precursor.
9. The method of claim 1, wherein the flexible polymeric article is elastomeric.
- 30 10. The method of claim 1, wherein the curing step comprises heating the precursor.

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11. The method of claim 1, wherein the curing step comprises heating the precursor to a temperature of at least about 65 °C.
12. The method of claim 1, wherein the curing step comprises heating the precursor for at least about 8 hours.
13. The method of claim 1, further comprising fastening a structural support to the flexible polymeric article.
14. The method of claim 1, wherein the flexible polymeric article is reusable.
15. A method, comprising:
  - providing a mold able to produce an article having a plurality of channels passing through the article, at least one of the plurality of channels having a cross-sectional dimension of less than 1 mm;
  - adding a precursor of a flexible polymer to the mold; and
  - applying pressure to the precursor while curing the precursor to produce a flexible polymeric article.
16. The method of claim 15, wherein the channel connects a first surface with an opposing second surface of the article.
17. The method of claim 15, wherein the mold comprises a photoresist surface.
18. The method of claim 15, wherein the mold comprises a semiconductor wafer.
19. The method of claim 15, wherein the mold is produced using photolithography.
20. The method of claim 15, wherein the precursor comprises PDMS precursor.
21. The method of claim 15, wherein the flexible polymeric article is elastomeric.



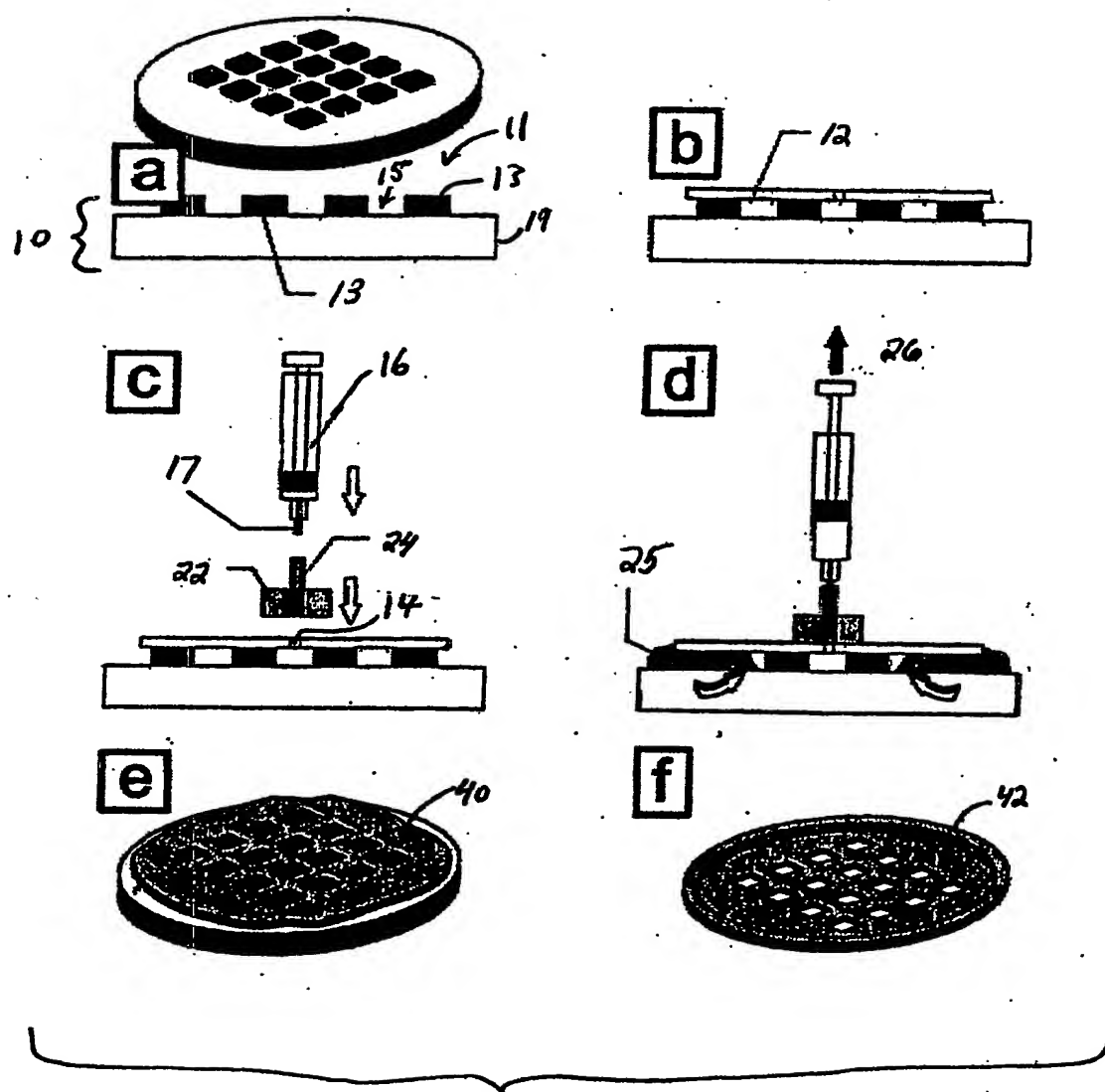
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22. The method of claim 15, wherein the curing step comprises heating the precursor.
23. The method of claim 15, wherein the curing step comprises heating the precursor to a temperature of at least about 65 °C.
24. The method of claim 15, wherein the curing step comprises heating the precursor for at least about 8 hours.
25. The method of claim 15, further comprising fastening a structural support to the flexible polymeric article.
26. The method of claim 15, wherein the flexible polymeric article is reusable.
27. A flexible polymeric article comprising a first surface, an opposing second surface, and a plurality of channels connecting the first surface with the second surface, at least one of the plurality of channels having a cross-sectional dimension of less than 1 mm and a length of less than about 25  $\mu$ m.
28. The flexible polymeric article of claim 27, wherein the flexible polymeric article is elastomeric.
29. The flexible polymeric article of claim 27, wherein the flexible polymeric article comprises PDMS.
30. The flexible polymeric article of claim 27, wherein the flexible polymeric article is reusable.
31. A method, comprising:  
treating at least a portion of a surface of a flexible polymeric article using oxygen-plasma treatment, the article having a plurality of channels

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passing through the article, at least one of the plurality of channels having a cross-sectional dimension of less than 1 mm.

- 5
- 32. The method of claim 31, wherein the flexible polymeric article is elastomeric.
  - 33. The method of claim 31, wherein the flexible polymeric article comprises PDMS.
  - 34. The method of claim 31, wherein the flexible polymeric article is reusable.

FIG. 1

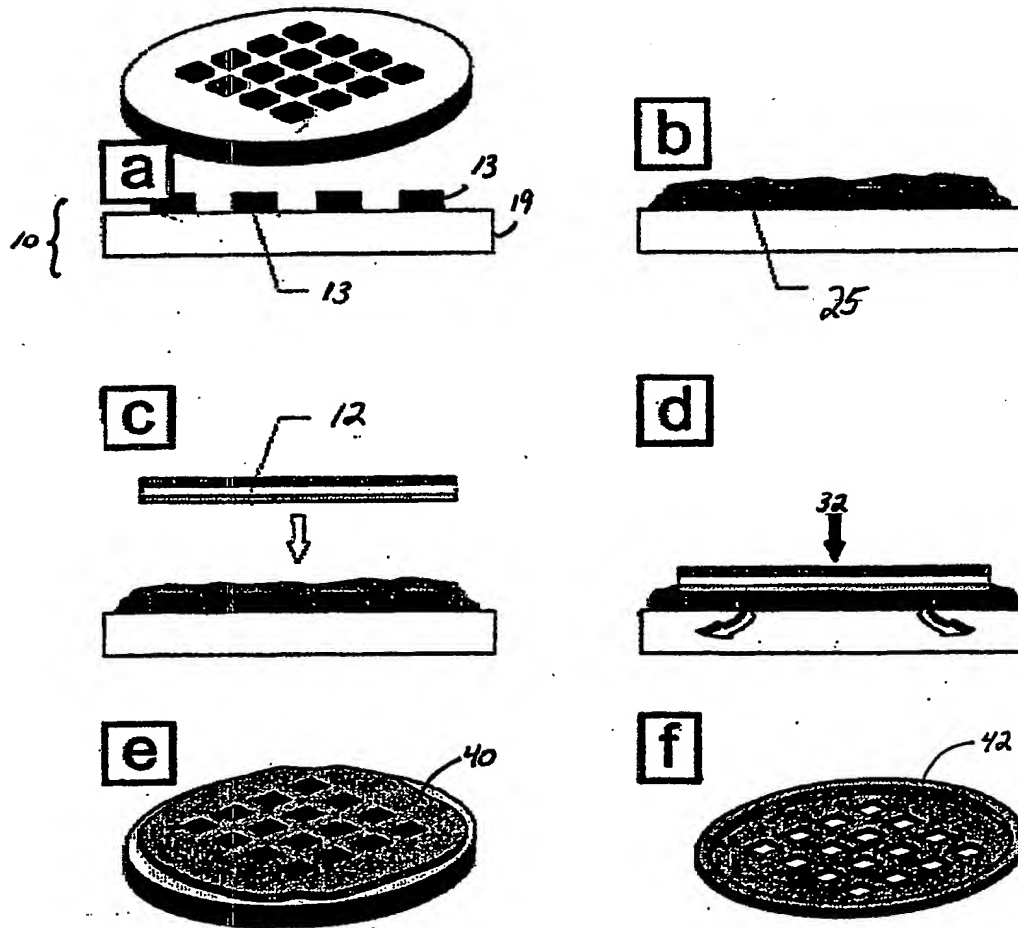


FIG. 2 :

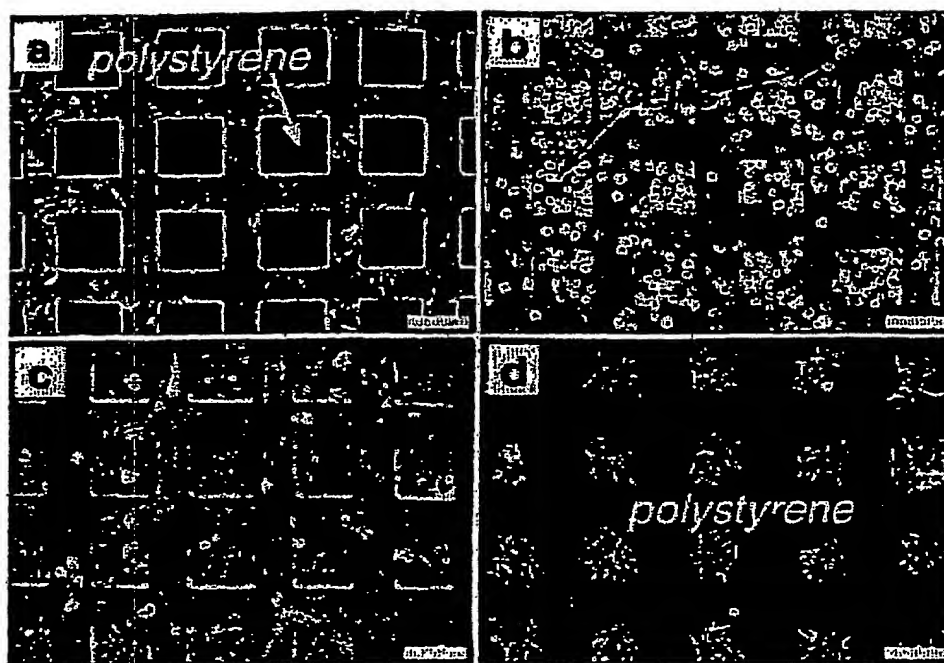


FIG. 3

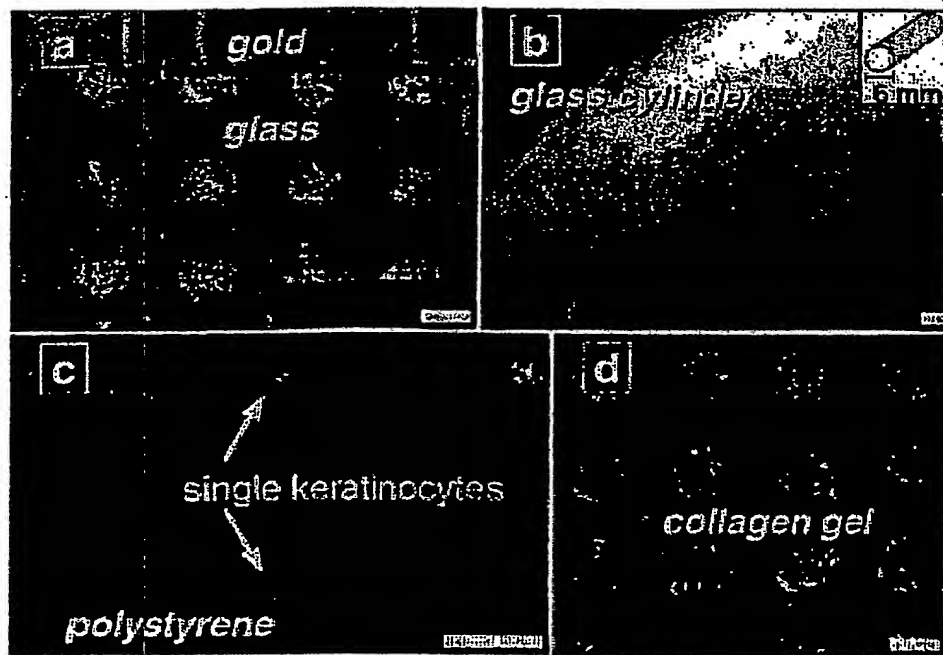


Fig. 4